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13. ABSTRACT *(Maximum 200*

Ceramide has emerged as an important intracellular regulator of cell growth and viability. In breast carcinoma cells, we find that tumor necrosis factor α (TNFα) causes prolonged and significant accumulation of ceramide, which precedes cell death. We have investigated the mechanism of ceramide formation and the mechanism of ceramide action, with specific emphasis on their interactions with proteases. Our studies lead us to define two phases of the apoptotic pathway: in the first, signaling phase, TNFα causes activation of proteases which lead to the accumulation of ceramide. In the second, execution phase, ceramide causes the activation of downstream death proteases as well as activation of the retinoblastoma gene product. Addition of exogenous ceramides causes simultaneously cell cycle arrest and cell death. These studies are beginning to identify a growth suppressor pathway in breast carcinoma cells and the results are beginning to interrelate important components involved in the apoptotic response.

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PI - Signature Sept 24/96 Date

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INTRODUCTION

Ceramide has recently emerged as an important endogenous candidate mediator of growth suppression; especially in response to cytotoxic agents and stress stimuli (1-3). Briefly, a number of extracellular agents have been found to induce ceramide formation. These include: Tumor Necrosis Factor alpha (TNFα), γ interferon, interleukin-1β, dexamethasone, Fas ligands, chemotherapeutic agents, and nerve growth factor (4). These agents cause the activation of membrane sphingomyelinases, which act on membrane sphingomyelin, causing the formation of ceramide. The addition of analogs of ceramide to cells causes either terminal cell differentiation, cell phrenescence, cell cycle arrest, or apoptosis(5). A role for the endogenous ceramide in mediating these biological effects in response to the above listed agents is further supported by the close association of formation of endogenous ceramide with these biological outcomes, the ability of exogenous ceramides to mimic these biologies, and the ability of agents that interfere with ceramide metabolism to modulate these responses(5,6).

The role of ceramide is further supported by recent insight into the mechanism of action of ceramide in vitro and in cells. Thus, in vitro ceramide has been shown to activate a serine/threonine protein phosphatase (7). This phosphatase is inhibited by okadaic acid, and okadaic acid appears to inhibit the effects of ceramide on apoptosis and growth suppression (8). Furthermore, an equivalent pathway has been demonstrated in yeast cells, whereby ceramide causes growth suppression of yeast (9). Genetic evidence was provided for the subunit composition of this phosphatase in yeast (10). Deletion of these subunits in yeast results in resistance to the activity of ceramide on growth suppression. Taken together, these studies suggest that ceramide in yeast works through activation of this phosphatase. Coupled with the inhibitor studies in mammalian cells, these studies would also suggest a role for this phosphatase in mediating the effect of ceramide on growth suppression.

In addition, ceramide has been shown to modulate a number of biochemical events in cells. For example, ceramide has been shown to activate the retinoblastoma gene product (Rb), down regulate the c-myc oncogene, inhibit phospholipase-D, modulate protein phosphorylation, activate protein kinases, and inhibit protein kinase Cα (8,11–14).

Major questions in this area of research center on: (1) What are the mechanisms involved in ceramide formation? (2) What are the mechanisms by which ceramide causes growth suppression? and (3) What is the precise role of ceramide in mediating growth suppression in response to TNFα, chemotherapeutic agents, and the other extracellular agents and stimuli that cause ceramide formation? The results presented in this document have aimed at addressing these issues. Results are beginning to clarify, especially the first two questions which, in turn, help address the third question.

BODY

Experimental Methods:

Quantitation of ceramide. Cells were seeded at 2 x 10^5 cells/ml in a 10 ml volume and then treated with TNFα 1.2 nM. Lipids were then collected according to the method of Bligh and Dyer (15). Cells were pelleted and washed once with phosphate buffered saline (PBS). They were then extracted with 3 ml of chloroform/methanol (1:2, v/v), and the monophase was mixed then 0.7 ml of water The organic phase was removed and transferred to a new tube and the samples were dried under N_2. Lipids were then resuspended in 1 ml of chloroform. Ceramide levels were measured using a modified diacylglycerol kinase assay (16) using external standards of diacylglycerol and ceramide. The radioactive spots corresponding to phosphatidic acid and ceramide-phosphate, the phosphorylated products of diacylglycerol (DAG) and ceramide respectively, were scraped into a scintillation vial. DAG and ceramide levels were normalized to lipid phosphate.

Proteolysis of PARP. For experiments analyzing PARP proteolysis, cells were seeded and treated, and at the indicated time points, cells were harvested by scraping in media followed by centrifugation at 4^0C. The cell pellet was then resuspended in 50 µl PBS and lysed with sample buffer (30 mM Tris-HCL [pH 6.8], 10 % glycerol, 6 %- b-mercaptoethanol, 4 % SDS) and boiled for 10 minutes. Protein concentrations were determined using the Bio-Rad assay. PARP proteolysis was then quantitated using Western Blot analysis: equal amounts of protein were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. PARP and its cleaved fragment were detected using a rabbit polyclonal antiserum at a dilution of 1:2000, and a goat anti-rabbit secondary antibody at a dilution of 1:5000. The signal was visualized by ECL (Amersham).

Western Blots for the retinoblastoma gene product (Rb). The status of Rb phosphorylation was determined using Western Blot analysis as described above. The hypophosphorylated Rb migrates faster than the phosphorylated species.

Transfection and expression of Bcl-2. Cells were transfected with either the vector pMEP4 alone or vector containing Bcl-2 using Lipofectamine. Cells were then selected with Hygromycin B1. Expression of Bcl-2 was confirmed by western blot analysis.

Tissue culture. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 0.2% sodium bicarbonate. G418 at 500 µg/ml was added to the CrmA cell line and its vector while hygromycin 150 µg/ml was added to the Bcl-2 cell line and its vector. Experiments were done in the absence of G418 or hygromycin. Cell viability was determined by the ability to exclude trypan blue.

Flow cytometry. Cell death was also verified by propidium iodide flow cytometry as described (17). Flow cytometry was also utilized to determine cell cycle progression and cell cycle arrest.

Ceramide Uptake. Cells were seeded at 2.5 x 10^5/well of a 6 well plate in a 2 ml volume of RPMI with 2% FBS and treated with $^{14}C_6$-ceramide at 20 µM. Cells were harvested by scraping at the indicated time points, and the radioactivity was counted in a scintillation counter.

Results.

1. *TNFα causes apoptotic cell death in MCF-7 cells as well as ceramide accumulation, which precedes cell death.* MCF-7 breast carcinoma cells were treated with TNFα, and ceramide levels and cell death were evaluated concomitantly at several time-points (Fig. 1). Ceramide levels did not change appreciably in the first 5 hours but were significantly increased between 7 and 9 hours and continued to increase with time; such that the levels of ceramide increased up to 400% by 20 hours.

It is becoming increasingly apparent this pattern of delayed accumulation of ceramide is most closely related to the apoptotic responses. While this raises the concern that ceramide accumulation is a consequence of death, studies with Bcl-2 (see below) negate this possibility. Cell death, as measured by the inability to exclude trypan blue, was not seen until 20 hours; occurring several hours following the increase in ceramide levels, indicating that ceramide accumulation occurs long before loss of membrane integrity.

In order to verify that cell death was occurring through induction of apoptosis, we assayed for cleavage of the 116 kDa poly(ADP-ribose) polymerase (PARP) polypeptide to a specific 85 kDa apoptotic fragment . This proteolytic cleavage has been shown to occur in apoptosis and to be mediated by Yama/CPP32/apopain or related proteases. It is now considered a hallmark of many apoptotic pathways, and it is a reliable parameter to distinguish apoptosis form necrosis. Teatment of MCF-7 cells with TNFα resulted in specific cleavage of PARP to the 85 kDa fragment (Fig. 2). Significant PARP cleavage did not occur until 12 hours after treatment with TNFα and was maximal by 25 hours. These results indicate that ceramide accumulation precedes by at least 3-4 hours one of the early signs of apoptosis, i.e. PARP cleavage.

2. *TNF causes cell cycle arrest and activates Rb.* When cells were treated with TNF, we observed a cell cycle phenotype such that treated cells were arrested in the G0/G1 phase of the cell cycle. Treatment with TNF and ceramide also caused dephosphorylation of Rb in a time dependent manner (Fig. 3). This was of great interest to us since: 1. dephosphorylated Rb appears to be the active species that induces a G0/G1 arrest; and 2. we have previously shown that ceramide causes an Rb-dependendt arrest in G0/G1 in Molt-4 cells. Thus, it is tempting to speculate that TNF causes a cell cycle arrest in a ceramide-dependent manner. Current studies aim at examining the effects of ceramide on cell cycle in MCF-7 cells.

3. *Involvement of proteases in ceramide formation.* The cowpox viral protein, CrmA, is a protease inhibitor which is known to inhibit, with variable efficiency, the ICE family of cysteine proteases (18). CrmA was utilized in our experiments to examine the relationship of these proteases to ceramide along the death pathway. MCF-7 cells stably expressing CrmA were treated with 2 nM TNFα or increasing concentrations of C_6-ceramide, a cell-permeable ceramide analog, and compared with control (vector) cells. CrmA offered almost complete protection from TNFα-induced cytotoxicity (Fig. 4). However, CrmA offered no protection from the cytotoxic effects of ceramide whereby cells died equally in the presence or absence of CrmA (Fig. 4). Therefore, CrmA appeared not to interfere with the downstream effects of ceramide.

Since endogenous ceramide elevation may drive the cell to die, we next examined whether CrmA interferes with ceramide generation. Endogenous ceramide levels were measured in response to TNFα in control and in CrmA-expressing MCF-7 cells. Ceramide increased significantly in vector cells at 18 and 24 hours (Fig. 5). However, CrmA-expressing cells treated similarly with TNFα showed no increase in ceramide levels. The results with CrmA suggest that proteases are involved in ceramide generation.

In order to determine whether the inhibition of ceramide accumulation was dependent on the ability of CrmA to inhibit proteases, we employed two approaches: first, we utilized a point-mutant of CrmA which has no antiproteolytic activity due to the substitution of Arg to Thr at amino acid 291 in the reactive site loop. In MCF-7 cells expressing this mutant, there was no significant inhibition of ceramide accumulation and no protection from TNFα-induced apoptosis (Fig 6). Second, we utilized the synthetic peptide Ac-YVAD-CHO, which, like CrmA, is a potent competitive inhibitor of ICE (18). Pre-treatment of cells with this peptide, resulted in significant inhibition of ceramide accumulation following TNFa (Fig. 6). Therefore, CrmA inhibits TNFα-induced apoptosis by acting at a point upstream of the generation of ceramide and this inhibition is dependent on its antiproteolytic activity.

4. *Further support that bcl-2 acts at the point downstream of ceramide but upstream of activation of the death proteases.* Since ceramide generation occurred in cells that eventually die in response to treatment with TNFα and not in the protected, CrmA-expressing cells, it became important to verify that this increase in ceramide levels, as well as the suppression of this increase by CrmA, were not nonspecific events correlating with cell death or survival, respectively. We utilized Bcl-2, another antiapoptotic molecule which has recently been shown to protect from ceramide-induced apoptosis (19). MCF-7 cells expressing Bcl-2 or vector controls were treated with TNFα or increasing concentrations of C_6-ceramide. Control cells died in response to both treatments but Bcl-2-expressing cells displayed resistance to TNFα-induced cell death as seen in cells expressing CrmA (Fig. 7). However, unlike CrmA-expressing cells, Bcl-2-expressing cells were resistant to ceramide-induced cell death. Additionally, generation of endogenous ceramide was nearly equal in both vector and Bcl-2 cells in response to TNFα, indicating that Bcl-2 does not interfere with ceramide generation (Fig. 8). Therefore, Bcl-2 functions at a point downstream of ceramide to inhibit apoptosis. More importantly, this delayed accumulation of ceramide is not a consequence of cell death since it is still observed in the viable Bcl-2 expressing cells. Thus, these data demonstrate that the elevation in ceramide is proximal to the biochemical and morphological changes of cell death.

5. *Ceramide causes activation of cell death proteases.* In order to determine the relationship between TNFα, ceramide, and subsequent PARP cleavage and apoptosis, it became imperative to study the effects and kinetics of ceramide on PARP cleavage. Consistent with results in Molt-4 cells (20), treatment of MCF-7 cells with ceramide resulted in cleavage of PARP (Fig. 9) and PARP cleavage following 4 hours of ceramide treatment was equivalent to that caused by 12-16 hours of TNFα treatment.

Next, it became important to determine the effect of CrmA or Bcl-2 expression on ceramide-induced PARP cleavage in MCF-7 cells. While both Bcl-2 and CrmA inhibited TNFα-induced PARP cleavage only Bcl-2 expression provided protection from ceramide-induced cleavage. PARP was cleaved after exogenous ceramide treatment despite high levels of expression of CrmA indicating that ceramide bypasses the CrmA target and activates a downstream protease capable of cleaving PARP (i.e. prICE) (Fig. 10). Notably, at the high levels expressed in this cell line, CrmA may partially inhibit this downstream protease which explains the slight inhibition of PARP cleavage in response to ceramide (Fig. 10). Therefore, in MCF-7 cells CrmA expression does not significantly interfere with activation of PARP cleavage by ceramide.

6. Uptake of exogenous ceramides by MCF-7 cells. By using ^{14}C-labeled ceramide and evaluating the kinetics of its uptake by MCF-7 cells at several time points, we found that ceramide was taken up slowly by these cells with maximal uptake reached only at 4 hours (Fig. 11). Therefore, the delay in PARP cleavage after ceramide treatment can be attributed to the delay in uptake of exogenous ceramide. Hence, these experiments support the hypothesis that ceramide accumulation following TNFα treatment may represent a trigger for PARP cleavage and apoptosis.

Relationship to Stated and Revised Goals.

Task 1:

A) We have conducted extensive studies on the effects of ceramide, tamoxifen, and TNF on growth and apoptosis. These results are detailed in the current and the previous reports. We have conducted studies with TNF on cell cycle arrest, and we have started studies with ceramide on cell cycle, Rb, and other parameters of cell cycle progression.

B) We have measured ceramide levels in response to TNF and tamoxifen. We are much more impressed by the changes with TNF, and therefore we are concentrating on this agent to define mechanism.

Task 2:

A) We have determined the effects of ceramide on Rb in Molt-4 cells. Current studies aim at evaluating the effects in MCF-7 cells. Preliminary data support this effect.

B) We have changed course in this task, as we have concentrated on the apoptotic pathway initially over the cell cycle pathway. To this end we have MCF-7 cells that express Bcl-2, CrmA, and mutant CrmA. Their effects on responses to TNF and ceramide are detailed in this current report.

Task 3: This task has been significantly revised as suggested in the previous report and as detailed in previous correspondence with Ms. Judy Pawlus and Dr. Patricia Modorw. We are still very interested in CAPP, but we have taken a biochemical and genetic line of investigation to study this phosphatase using tissues for purification and yeast cells for genetic studies. Instead, we have concentrated in the MCF-7 cells on the mechanisms involved in ceramide-induced apoptosis. To this end we have defined the effects of ceramide on proteases and the role of these proteases in mediating the effects of ceramide on apoptosis as discussed in this report. Future work will concentrate on the mechanisms involved in regulation of the proteases by ceramide, TNF, and chemotherapeutic agents.

CONCLUSIONS

Studies in the first year of the proposal demonstrated the operation of the sphingomyelinase-ceramide pathway in MCF-7 cells which is activated by tamoxifen and TNFα. Because of several considerations, we chose to concentrate primarily on TNFα. First, the magnitude and kinetics of ceramide formation in response to TNFα have been more consistent and more significant with TNFα than with tamoxifen. Once we elucidate the mechanisms of TNF, we will go back and examine the activities of other chemotherapeutic agents as well as tamoxifen. Second, there has been an explosion of research in determining components of the apoptotic pathway in response to TNFα (21–23). This has made it imperative for us to determine the relationship of sphingomyelinase and ceramide to these pathways. Third, tamoxifen may exert other activities in cells, such as inhibition of protein kinase C, as well as its anti-estrogenic activities which may make interpretation of studies on ceramide more difficult to interpret.

Several conclusions can be derived from these studies which have accumulated over the first two years of the work. First, TNFα causes apoptosis in MCF-7 cells. This is substantiated by the ability of TNFα to cause PARP cleavage. This supports observations made with TNFα in several cell lines by several groups over the last few years (21,23,24). Second, TNFα causes significant cell cycle arrest in MCF-7 cells. This is a novel finding for TNFα. It is also supported by the ability of TNFα to cause dephosphorylation of Rb. Third, ceramide causes both apoptotic cell death as well as cell cycle arrest in MCF-7 cells. Fourth, the studies with CrmA and protease inhibitors (inhibitors of the ICE subfamily of proteases) demonstrate the involvement of proteases in the accumulation of ceramide. Fifth, these studies clearly demonstrate the ability of ceramide to activate PARP proteases, which have been dubbed as cell death proteases because of their intimate connection with apoptotic cell death. Sixth, our studies show that bcl-2 acts downstream of ceramide formation but upstream of activation of these cell death proteases, since overexpression of bcl-2 can inhibit the effect of ceramide on the activation of these proteases.

Taken together, these studies are providing significant insight into interrelationships between various components involved in initiating, signaling, modulation, and execution of the apoptotic pathway. Thus, we are beginning to elucidate two distinct classes of proteases, those involved in initial signaling from TNFα and those involved in the execution phase of apoptosis. Our studies suggest that ceramide is interpolated between these proteases. Furthermore, bcl-2 appears to act preferentially on the downstream proteases and not on the upstream proteases.

Future directions aim at further understanding of this apoptotic pathway, determining more precisely the role of ceramide in the apoptotic pathway, defining the ability of various chemotherapeutic agents to induce ceramide formation, and delineating the role of ceramide in apoptotic cell death and growth suppression.

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APPENDIX (Figures 1-11)

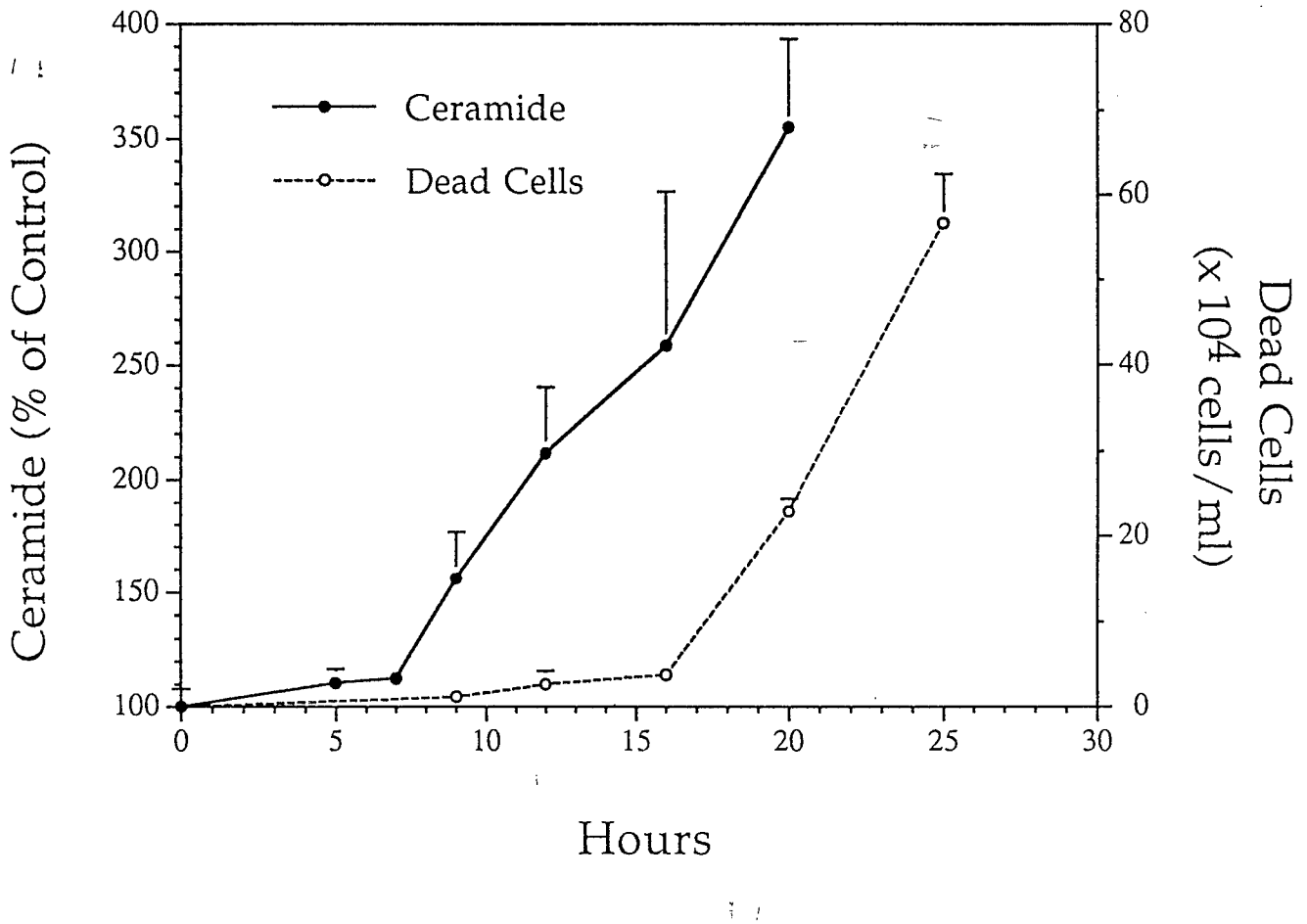

Figure 1. TNF causes cell death and ceramide accumulation in MCF-7 breast carcinoma cells. MCF-7 cells were treated with 2 nM TNF and ceramide and cell death were quantitated as described in the text.

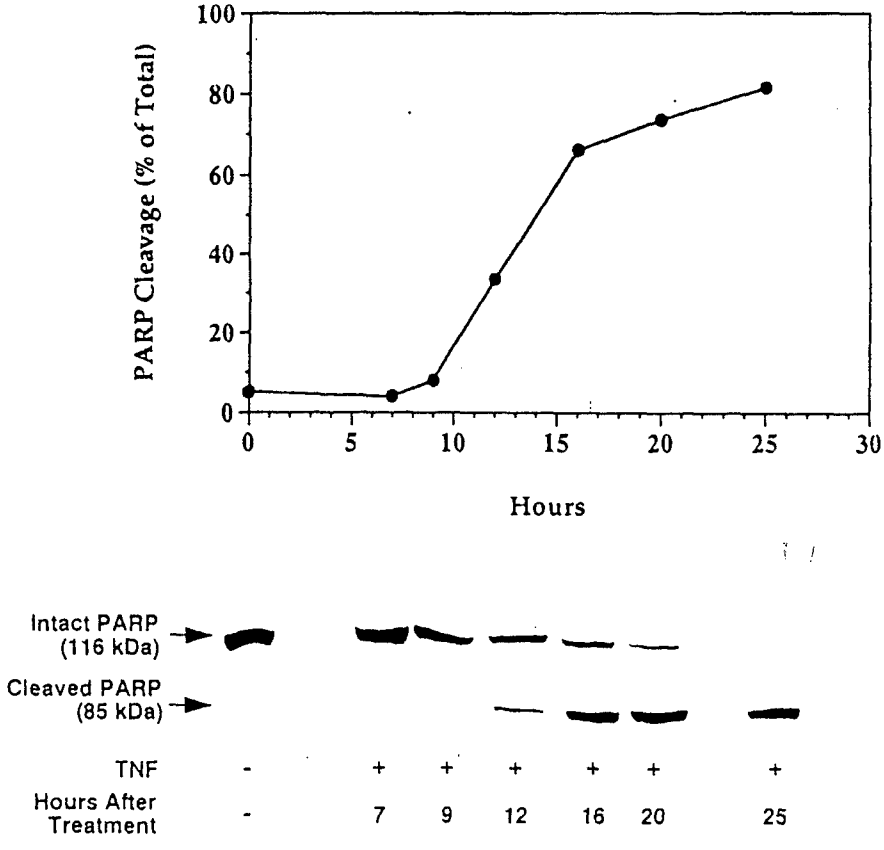

Figure 2. TNF causes apoptosis of MCF-7 cells as deteremined by proteolysis of PARP. Shown is a time course for PARP proteolysis in cells treated with 2 nM TNF.

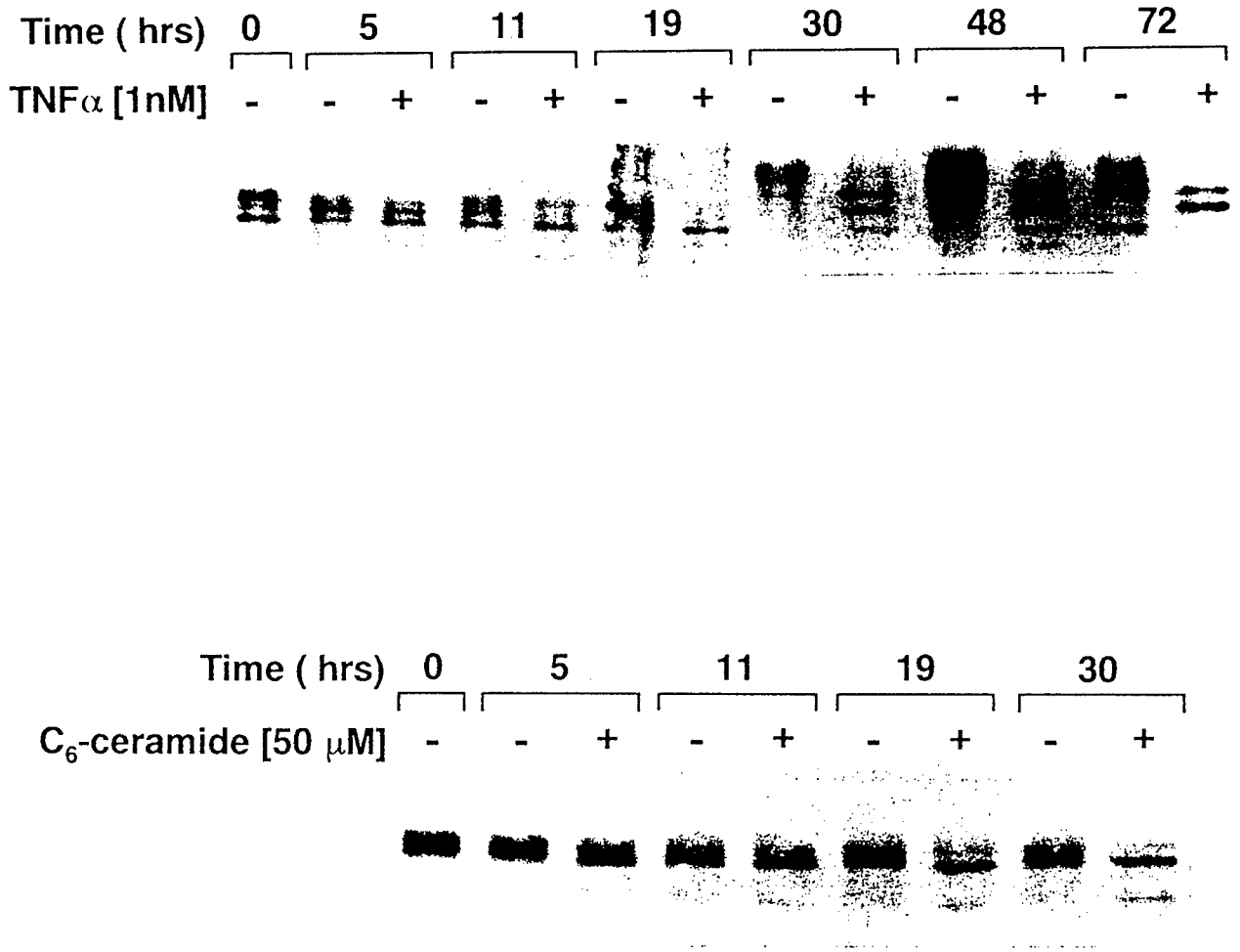

Figure 3. TNF and ceramide cause dephosphorylation of Rb in MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of TNF or ceramide. Proteins were harvested at the indicated time points and Rb phosphorylation/dephosphorylation was evaluated by Wester Blotting.

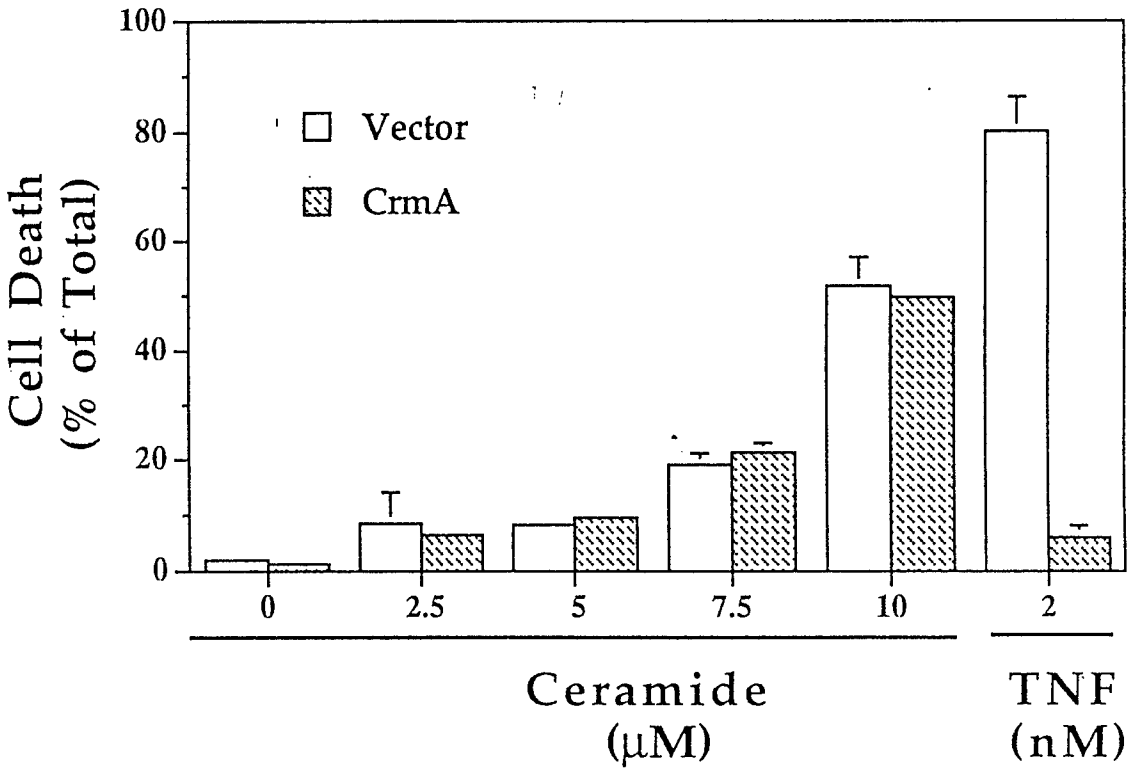

Figure 4. CrmA protects from TNF but not from ceramide induced cell death. MCF-7 cells were treated with the indicated concentrations of ceramide or TNF and cell death was evaluated as described in methods. CrmA-expressing cells were protected from TNF when compared to vector cells.

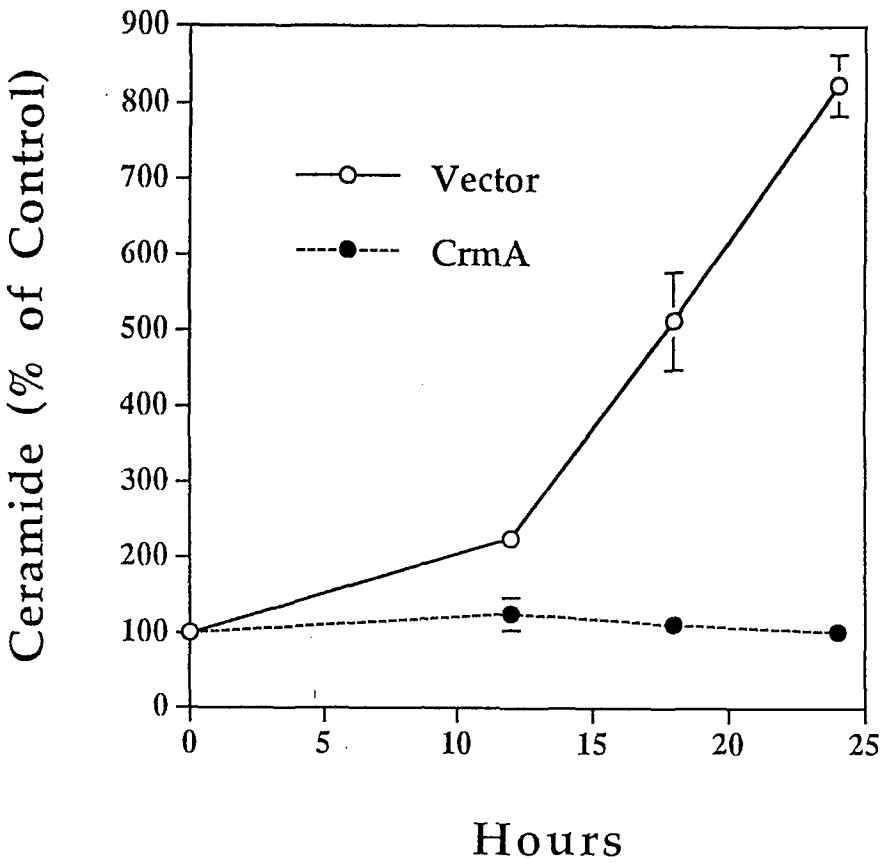

Figure 5. CrmA inhibits ceramide formation. MCF-7 cells were treated with 2 nM of TNF and ceramide levels were quantitated as described in methods.

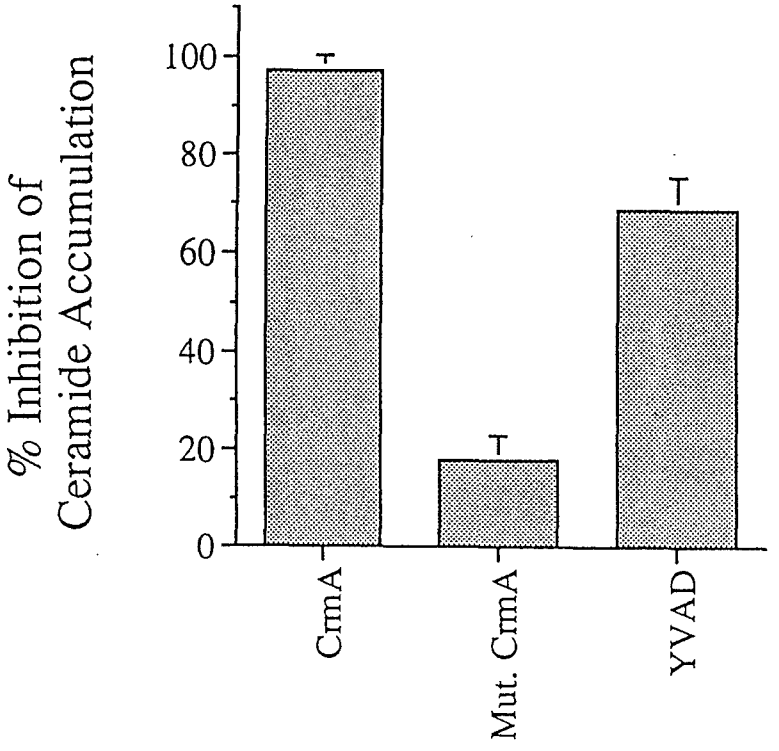

Figure 6. Role of proteases in the action of CrmA. Cells were transfected with either wild type CrmA or with a mutant that does not inhibit proteases. Cells were then treated with 2 nM TNF, and cell death was evaluated. Only in cells expressing wild type CrmA was ceramide accumulation inhibited. Also, cells treated with the protease inhibitor, YVAD, ceramide levels were also suppressed.

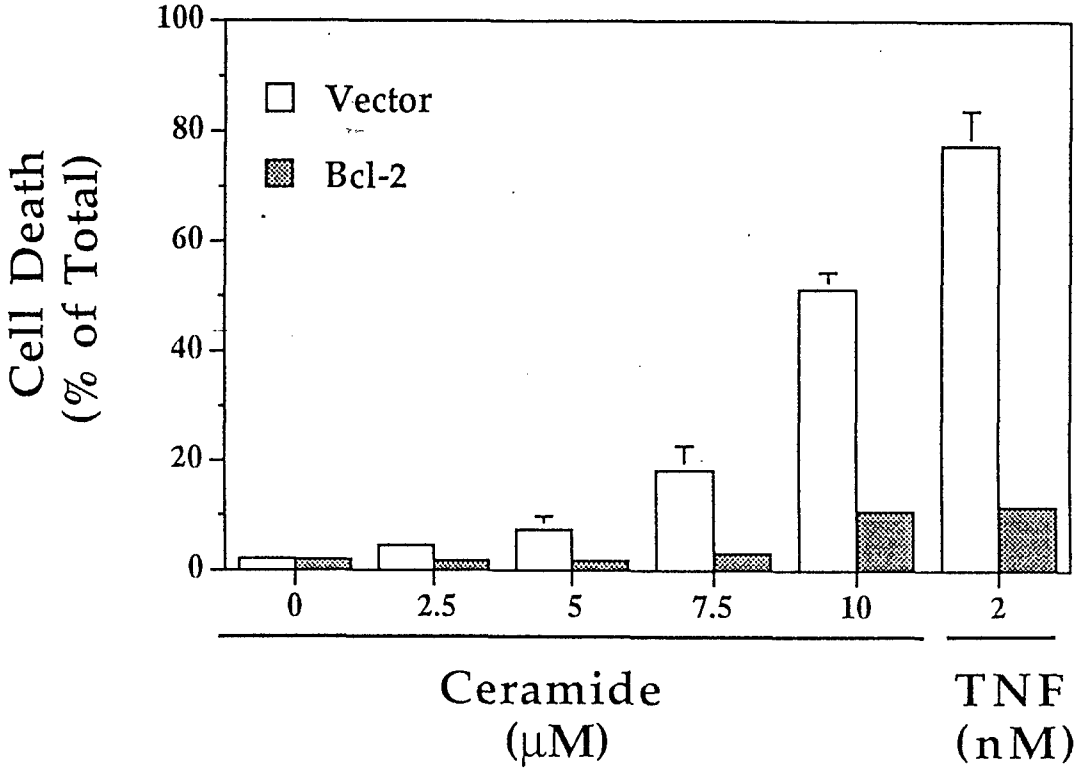

Figure 7. Bcl-2 inhibits TNF and ceramide-induced death of MCF-7 cells. Cells overexpressing Bcl-2 were protected from death in response to either TNF or ceramide.

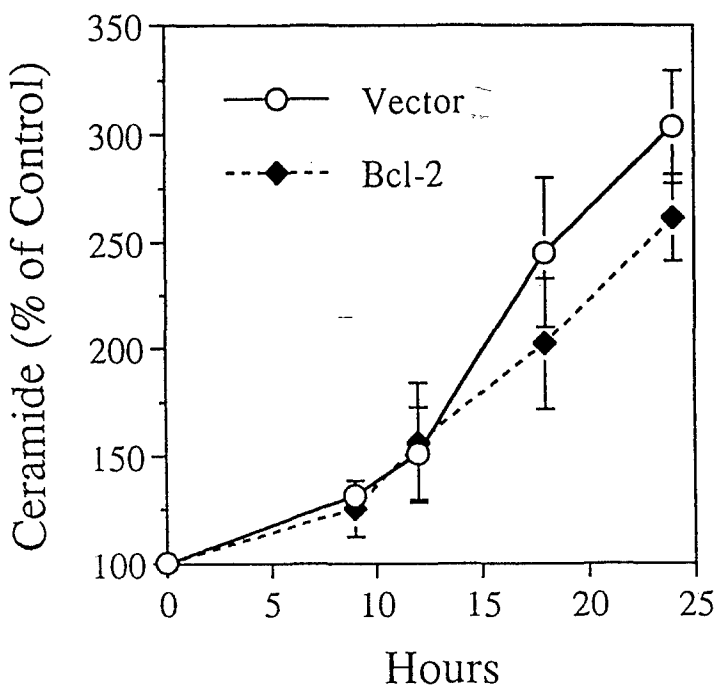

Figure 8. Bcl-2 does not suppress ceramide levels in cells treated with TNF.

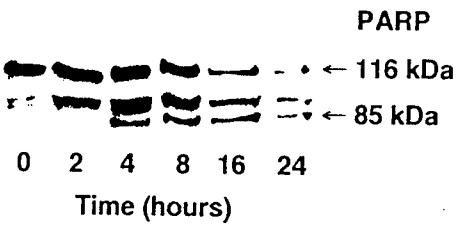

Figure 9. Ceramide induces proteolysis of PARP. MCF-7 cells were incubated with 10 μM C6-ceramide, and PARP proteolysis was evaluated at the indicated time points.

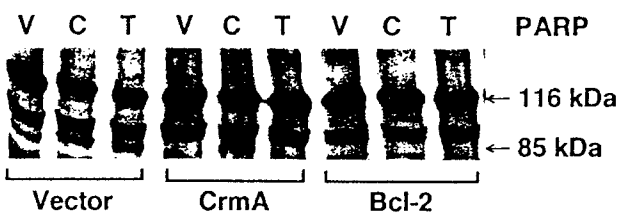

Figure 10. Bcl-2 inhibits PARP proteolysis in response to TNF(T) and ceramide (C), but CrmA inhibits only the effects of TNF. V= vehicle.

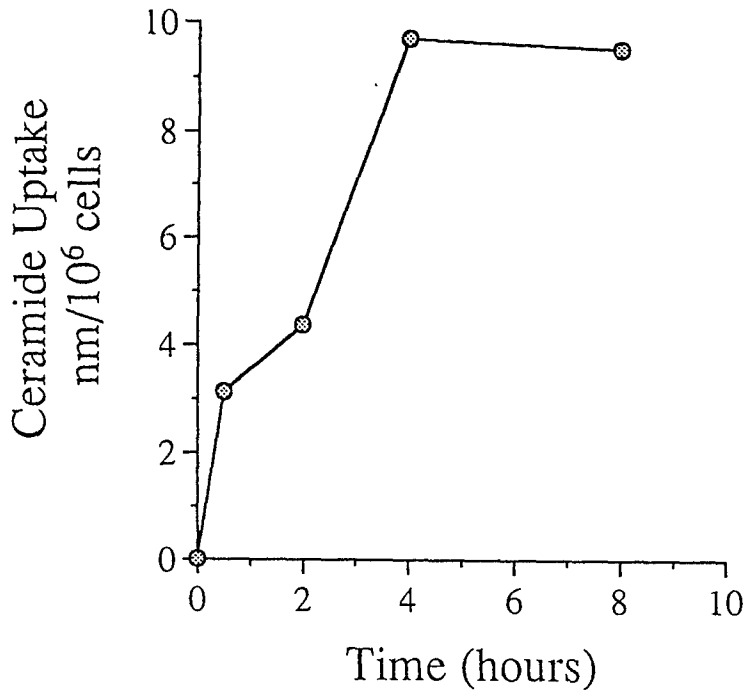

Figure 11. Uptake of ceramide by MCF-7 cells. MCF-7 cells were incubated with radiolabeled C6-ceramide, and the amount of ceramide taken up by cells was measured at the indicated time points.

Recd 2/25/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Feb 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical report written for Grant DAMD17-94-J-4266 and DAMD17-94-J-4301. Request the limited distribution statement for Accession Document Numbers ADB222576, ADB221779, and ADB235519, be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management